on a 10% gel and then transferred to nitrocellulose using a semi-dry electroblotter. The nitrocellulose was blocked and loaded into a Multi-Screen immunoblotting apparatus (Bio-Rad). Pooled serum from each group of immunized mice was diluted and then incubated in separate wells of the apparatus. The blot was developed using an alkaline phosphatase-conjugated goat antibody to mouse IgG (Kirkegaard & Perry Labs, Gaithersburg, Md.) to identify the presence of BoNT/A-specific antibody.

Construction and expression of BoNT/A gene fragments. The BoNT/A gene was subcloned into overlapping fragments ranging in size from ~300-600 base pairs by using PCR. The primers encoded flanking restriction sites that permitted convenient insertion into the expression vector used, and allowed transcriptional and translational read-through of the amplified fragments to occur (Table 1). Plasmid vector pMTD74 was used to express the amplified BoNT/A gene fragments in E. coli. Insertion of the PCR-amplified fragments into the expression vector pMTD74 resulted in translational fusion to the A2 peptide of cholera toxin (CtxA2) (8). The fragments were fused to CtxA2 to provide a vaccine for administration mucosally. The presence of BoNT/A-specific protein was determined by immunoblotting analysis, using polyclonal horse antiserum to BoNT/A, and by comparison of the predicted size of the truncated protein to its actual size. Fusion to CtxA2 increased the predicted size of the truncated BoNT/A proteins expressed by an additional 5.4 kDa, but it did not appear to affect their ability to be produced. By expressing overlapping segments of the toxin, all potential linear epitopes were encoded. BoNT/A is post translationally cleaved into the light (L) and heavy (H) chains which are joined together by a disulfide bond. The position of each fragment within BoNT/A is indicated by the chain it was derived from (L or H), followed by the amino acid residues of BoNT/A encoded.). The T7 promoter expressed these proteins at high levels in E. coli. The BoNT/A-specific proteins were expressed primarily in the form of inclusion bodies that could be isolated by differential centrifu-

gation upon lysis of the cells.

Purification of BoNT/A proteins. While the crude lysates containing the BoNT/A proteins were initially used for immunization of mice, it was found preferable to use at least partially purified materials to provide improved tolerance and to effectively produce strong, specific antibody response. For these reasons, the lysates containing the truncated BoNT/A proteins were subjected to purification by preparative SDS-PAGE and then used for immunization of mice. Preparative SDS-PAGE provided a convenient method of both solubilizing and separating the BoNT/A proteins from the majority of other contaminants present in the lysates. Although the BoNT/A proteins were not always purified to homogeneity, they were highly enriched. Furthermore, the BoNT/A proteins remained soluble after the SDS was removed, which facilitated the administration of these proteins to mice.

Immunogenicity of BoNT/A proteins. Mice were immunized i.p. with the truncated BoNT/A proteins emulsified in Ribi™ adjuvant. The mice were immunized at 2-week intervals, and one week after the last immunization, their serum was analyzed for the presence of antibody to BoNT/A. Since BoNT/A can be separated by SDS-PAGE into a 50 kDa light chain and 100 kDa heavy chain, immunoblotting analysis was used to evaluate whether the antibody elicited by each truncated protein reacted with the appropriate chain. Optimal antibody responses were observed in mice after the fourth dose. All of the truncated proteins were able to elicit an antibody response except $H_{1078-1220}$. Although this fragment was non-immunogenic, it was highly antigenic when reacted with polyclonal horse antiserum to BoNT/A. Unlike the crude lysates used for immunization previously, the purified proteins were well-tolerated and could be repeatedly administered to the mice. In addition, the purified proteins were able to elicit an BoNT/A-specific antibody response in mice. This difference in the immunogenicity of the crude lysates cannot be accounted for by the lack of BoNT/A-specific protein, since the lysates used for immunization were known to contain appreciable quantities of

truncated protein.

Protective efficacy of BoNT/A proteins. Two weeks after the final immunization, each mouse was challenged i.p. with 2 lethal doses of BoNT/A (2 $MIPLD_{99}$). This dose was chosen for initial screening to observe any potential ability of the proteins to elicit protective immunity. As shown in Table 3, only two proteins protected the majority of animals from death. Both of these fragments were derived from the heavy chain and encoded amino acid residues $H_{455-661}$ and $H_{1150-1289}$.

$H_{455-661}$ of serotype A neurotoxin is the sequence

H_3N-IKVNN WDLFF SPSED NFTND LNKGE EITSD TNIEA AEENI SLDLI QQYYL TFNFD NEPEN ISIEN LSSDI IGQLE LMPNI ERFPN GKKYE LDKYT MFHYL RAQEF EHGKS RIALT NSVNE ALLNP SRVYT FFSSD YVKKV NKATE AAMFL GWVEQ LVYDF TDETS EVSTT DKIAD ITIII PYIGP ALNIG NMLYK DDFVG ALIFS GA-COOH

and $H_{1150-1289}$ of serotype A neurotoxin is the sequence

H_3N-LNSSL YRGTK FIIKK YASGN KDNIV RNNDR VYINV VVKNK EYRLA TNASQ AGVEK ILSAL EIPDV GNLSQ VVVMK SKNDQ GITNK CKMNL QDNNG NDIGF IGFHQ FNNIA KLVAS NWYNR QIERS SRTLG CSWEF IPVDD-COOH.

Although some of the other truncated proteins appeared to provide partial protection at the challenge dose initially used, none were as definitive as $H_{455-661}$ and $H_{1150-1289}$. Rechallenge of the survivors with 2 $MIPLD_{99}$ of BoNT/A resulted in the death of all mice except those immunized with the two protective fragments. To confirm these results, separate groups of mice were immunized with fragments $H_{455-661}$ and $H_{1150-1289}$ as before and then challenged with 10 $MIPLD_{50}$. The survival rate for mice immunized with $H_{455-661}$ and $H_{1150-1289}$ at this challenge dose was determined to be 87.5% and 60.0%, respectively.

TABLE 3. Immunogenicity and protective efficacy of the truncated BoNT/A proteins

Protein Segment[a]	Immuno-Blot[b]	Number of Survivors[c]	% Survival
$L_{4\ 128}$	+	1/10	10.0
$L_{126-271}$	+	0/8	0.0
$L_{247-465}$	+	0/9	0.0
$H_{455\ 661}$	+	7/9	77.8
$H_{650-808}$	+	0/5	0.0
$H_{780\ 939}$	+	2/7	28.6
$H_{915-1059}$	+	0/8	0.0
$H_{982\ 1123}$	+	1/9	11.1
$H_{1078\ 1220}$	-	0/5	0.0
$H_{1150-1289}$	+	6/8	75.0

[a] Amino acid residue number of the light (L) chain and the heavy (H) chain.

[b] CB6F1 mice were immunized i.p. with four doses of each protein at 2-week intervals. One week after the last dose, the mice were bled and the serum was analyzed by immunoblot for the presence of antibody specific for BoNT/A.

[c] Number of survivors/total number 4 days after challenge with 2 $MIPLD_{99}$ of BoNT/A.

Immunoblotting analysis was used to detect the presence of BoNT/A-specific antibody in the immunized mice for several reasons. First, the sensitivity of this method maximized the probability of detecting the presence of any fragment-specific antibody, regardless of whether it was directed towards a linear or a conformational epitope. Second, by separating BoNT/A into its heavy and light chains, this procedure also permitted the chain specificity of the antibody to be confirmed. By this method, all fragments were able to elicit an antibody response, except $H_{1078-1220}$.

Although most of the BoNT/A fragments were able to elicit antibody, only two were clearly able to confer protective immunity (Table 3). The protective efficacy of $H_{455-661}$ and $H_{1150-1289}$ correlates well with the potential functional role of these domains. The N-terminal half of the heavy chain (H_N) of BoNT/A, from which $H_{455-661}$ was derived, has been shown to be important in productive binding and internalization of the toxin to the cell. The C-terminal half of the heavy chain (H_C), from which $H_{1150-1289}$ was derived, has been associated with the initial binding of the toxin to the cell. If these functions are encoded by either fragment, then antibody specific to these domains would be predicted to interfere with the binding and/or internalization of BoNT/A. This, in turn, would prevent intoxication of the cell. The location of these protective domains on the extreme N- and C-terminal ends of the heavy chain suggest that important functional roles may also be encoded by these fragments. We are currently exploring this possibility.

The light chain fragment $L_{126-271}$ did not confer protection even though it elicited an antibody response (Table 3). This fragment encodes a highly conserved histidine-rich motif characteristic of zinc-dependent metalloproteases, such as BoNT/A. Although unproven, antibody directed to this region may block the enzymatic activity of BoNT/A. The inability of $L_{126-271}$ to protect suggests that the antibody elicited by this fragment may not have been directed towards epitopes involved in the

enzymatic activity of the light chain.

Studies with MAbs suggest that many of the antibody determinants of BoNT/A may be conformationally sensitive, and there is evidence to suggest that BoNT/A is an oligomeric protein. If BoNT/A is indeed oligomeric, then it is possible that some epitopes are formed by the interaction of adjoining subunits. Alternatively, linear-distant parts of the toxin molecule may come together when folded to form epitopes, as appears to be the case for the light chain. Comparison of the amino acid sequence of these fragments with the amino acid sequence of similar regions from the other serotypes did not show any significant homology. A cocktail of recombinant proteins containing amino acid sequences from analogous domains other serotypes ($H_{455-661}$ and $H_{1150-1289}$) should be prepared using the methods of the invention to provide immune protection against more than one serotype of organism.

The entire domains of $H_{455-661}$ and/or $H_{1150-1289}$ need not be used to provide a vaccine. However, at least 100 amino acids from one of the domains of any serotype should be used to provide sufficient antigenicity and immunoprotection.